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TITLE: Design and Testing of Bi-Functional, P-Loop-Targeted MDM2 Inhibitors

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14. ABSTRACT

Our proposal is to design and evaluate a novel class of bifunctional MDM2 inhibitors, based on the discovery that nucleotides can bind to the P-loop of MDM2 and cause its relocalization to the nucleolus. Such bifunctional compounds will be designed to target MDM2, but not other P-loop-containing proteins. This approach represents a new strategy for the inhibition of MDM2 function and the treatment of breast cancer. During the second year of this grant we have used the cloned, expressed, and purified GST-fused Mdm2 wild-type and C-terminally deleted RING domain protein to continue to test all commercially and privately available ATP analogs (including fluorescent analogs) for binding to Mdm2 and have further insight into the features of ATP required for binding to Mdm2. We have also (1) determined that crosslinking to 8-azido ATP inhibits ATP binding and E3 ligase activity; (2) developed high-throughput auto-ubiquitination assay to discover Mdm2 ligase inhibitors (3) developed a high-throughput docking assay based on Mdm2's RING domain structure and (4) developed a high-throughput compatible luciferase-based competition assay for compounds that bind to Mdm2.

15. SUBJECT TERMS
MDM2, p53, ring, ubiquitin ligase, combinatorial chemistry, high-throughput screening, chemical biology, chemical genetics, synthetic lethality

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Introduction

The Mdm2 protein plays essential roles in negatively regulating the p53 tumor suppressor protein. Mdm2 has been found to be upregulated in a significant number of breast cancers. The Mdm2 RING domain possesses a cryptic nucleolar localization signal sequence and a nucleotide-binding P-loop motif. These two motifs are unique to the Mdm2 RING and are not present in the myriad other RING domains encoded in the human genome. Nucleolar relocalization of Mdm2 causes p53 stabilization and activation, and nucleotide binding to the Mdm2 RING domain facilitates Mdm2 nucleolar localization.

Mdm2 has been found to play an essential role in negatively regulating the p53. In turn, the p53 protein binds to the promoter of Mdm2 and activates Mdm2 transcription. The importance of Mdm2 in p53 down-regulation has also been shown by genetic analysis: loss of *mdm2* in mice results in early embryonic lethality, but deletion of *p53* with *mdm2* rescues the lethal phenotype.

There is mounting evidence for a pro-oncogenic role for Mdm2 that is independent of p53 in both murine models and in human tumor cohort studies. Therefore the novel inhibitors that we plan to develop may have broader impact than activation of p53.

The design of genetically-targeted anti-tumor agents is an important new strategy in cancer drug discovery. However, it is often difficult to identify the specific proteins that should be targeted for maximal clinical benefit and to develop small molecules that target these proteins. We have identified an opportunity to develop a potentially powerful new class of compounds targeted against breast cancers with a specific genetic modification. We propose to develop genetically targeted small molecules that will selectively eradicate breast cancer cells containing amplified or up-regulated Mdm2.

Project Progress - Body

Previously, we have cloned, bacterially expressed, and affinity purified GST-fused Mdm2 wild-type RING domain as well as a C-terminally deleted Mdm2 RING domain protein that does not oligomerize in solution and exhibits dramatically increased solubility when compared to similarly expressed and purified wild-type Mdm2 RING. This protein is ideal for development of a high-throughput assay due to its high bacterial yield and excellent solubility.

Using this protein, we have performed *in vitro* filter binding competition assays with ^{32}P γ ATP to test all commercially available ATP analogs, including fluorescent analogs and several new analogs (Figure 1,a), for binding to the Mdm2 RING. These binding assays have yielded a detailed map of the features of ATP required for binding to the Mdm2 RING domain (Figure 1,b). We have also found that MdmX, an Mdm2 family member with high sequence homology, binds adenine-base nucleotides with similar affinity as Mdm2 (Figure 1,c). This information is being used in an *in silico* screen for compounds that are likely to bind to Mdm2 for further testing in a high-throughput screen.

8-azido ATP, an ATP analogs that binds to the Mdm2 RING domain with similar affinity as unlabled ATP *in vitro* (Figure 2,a), can be crosslinked to Mdm2 in a UV-dependent manner. Mdm2 RING domain that is crosslinked to 8-azido ATP can no longer bind ^{32}P γ ATP, suggesting that the crosslinked ATP is specifically occupying the correct ATP binding site of the protein (Figure 2,b). Additionally, Mdm2 RING that has been crosslinked to 8-azido ATP shows decreased ability to polymerize ^{32}P-ubiquitin *in vitro*, suggesting that ATP binding directly inhibits the E3 ligase activity of Mdm2 RING (Figure 2,c). This data suggests that ATP-like compounds that bind selectively to the Mdm2 RING would be possibly inhibitors of E3 ligase activity.

We have also made progress developing a high-throughput screen for compounds that bind to Mdm2. We first attempted to optimize a commercial FRET assay (Lance, Perkin Elmer) that would detect ATP binding to Mdm2 in a high-throughput compatible format (Figure 3,a). In this assay, biotinylated ATP, bound to a streptavidin molecule with a acceptor fluorophore binds to GST-Mdm2 that has been labaled with an anti-GST antibody tagged with the donor fluorophore. Fluoresence output is disrupted if a test compound displaces the biotinylated ATP from the Mdm2 binding site, signaling a compound that binds to Mdm2. We determined that biotinylated ATP binds to the Mdm2 RING with similar affinity as unlabeled ATP (Figure 3,b) and that this binding is not disrupted in the presence of streptavidin-APC (Figure 3,c). However, we were unable to detect any fluorescence output with GST-Mdm2, although our positive and negative controls were successful (Figure 3,d). In order to optimize our reaction conditions, we directly biotinylated GST and GST-Mdm2 RING, confirming labeling using an anti-biotin immunoblot (Figure 3,e). We were able to detect fluorescence output from biotinylated GST, but not GST-Mdm2 RING (Figure 3,f), suggesting that the Mdm2 RING obscures the epitope of the fluorescently labeled anti-GST antibody.

We are currently in the process of optimizing a luciferase-based competition assay to detect binding to Mdm2 in a high-throughput format. In this assay, GST-Mdm2 that has been adsorbed a 384-well plate is incubated with a small concentration of ATP

and a test compound. If this compound binds to the ATP-binding site of Mdm2, it competes off the ATP. Changes in free ATP concentration are determined by addition of luciferase to the soluble portion of the reaction, resulting in an increased luciferase signal if the compound binds Mdm2. We have determined that, under our binding conditions, luciferase is a sensitive indicator of ATP concentration (Figure 4,a). We also have shown in preliminary trials that this assay may be able to indicate binding to Mdm2 using ribavirin, a compound that we have shown previously binds Mdm2 (Figure 4,b). This assay has the advantage of being both sensitive and specific. It will only detect compounds that bind specifically to the ATP-binding site of Mdm2, and is capable of detecting compounds that bind with an affinity of less than 10-fold below that of ATP. However, we are still in the process of optimizing this assay. At present, it lacks the sensitivity to definitively distinguish between ATP-binding and non-binding compounds outside of standard error (Figure 4,b), but we will continue to optimize assay conditions. We may also explore fluorescence polarization-based assays using fluorescently labeled analogs that we have found to bind to the Mdm2 RING with similar affinity as ATP (Figure 1).

In an effort to find compounds that inhibit Mdm2 ligase activity, we have succeeded in developing a high throughput *in vitro* ubiquitination assay which makes use of the Mdm2 RING domain's ability to auto-ubiquitinate(Figure 5.). The assay involves the reconstitution of the ubiquitination pathway using biotin-labeled ubiquitin. In the assay, E1 enzyme is used to activate the labeled substrate and transfer it to an ubiquitin carrier protein, UbcH5c. The substrate bound E2 is then used by GST-tagged Mdm2 RING domain to auto-ubiquitinate. Detection of ubiquitined Mdm2 is done by transferring the reaction to a streptavidin-coated plate, washing and detecting bound Mdm2 by incubating with HRP-linked α-GST antibodies then detecting luminescence by adding ECL reagent.

Also, as a new approach to find hit compounds, we have developed a virtual docking assay, which was made possible by the recent publication of the structure of the MDM-2 RING domain. This computational approach to drug discovery makes use of the existence of an available crystallographic or NMR structure of the target by docking small molecules into the ligand binding site of the protein. We used the monomeric form of the published RING domain structure (Kostic et al. 2006.) as a docking target. Based on previous mutational studies on the RING domain (Poyurovsky et al. 2003.) and molecular dynamics simulations we predicted the ATP binding site to be in an exposed surface with K454 of the RING domain coordinating the beta- and gamma-phosphates of ATP (Figure 6.).

For this study, we screened 239 199 compounds available from commercial vendors using FRED2.1 (Openeye Scientific) for docking and OMEGA 1.8 (Openeye Scientific) for conformer generation. Each compound was represented by 200 conformers in the screening dataset to properly explore conformational space when docking. The top scoring 500 compounds will be ordered and screened in the previously mentioned luciferase assay.

Key Research Accomplishments

Our proposal is to design and evaluate a novel class of bifunctional MDM2 inhibitors, based on the discovery that nucleotides can bind to the P-loop of MDM2 and cause its relocalization to the nucleolus. Such bifunctional compounds will be designed to target MDM2, but not other P-loop-containing proteins. This approach represents a new strategy for the inhibition of MDM2 function and the treatment of breast cancer. During the second year of this grant we have used the cloned, expressed, and purified GST-fused Mdm2 wild-type and C-terminally deleted RING domain protein to continue to test all commercially and privately available ATP analogs (including fluorescent analogs) for binding to Mdm2 and have further insight into the features of ATP required for binding to Mdm2. We have also (1) determined that crosslinking to 8-azido ATP inhibits ATP binding and E3 ligase activity; (2) Developed high-throughput auto-ubiquitination assay to discover Mdm2 ligase inhibitors (3) dveloped a high-throughput docking assay based on Mdm2-s RING domain structure and (4) dveloped ahigh-throughput compatible luciferase-based competition assay for compounds that bind to Mdm2

Reportable Outcomes

Posters

13th Annual p53 Workshop, May 20-24, 2006. Columbia University, New York, NY, USA.

Deconstructing Nucleotide Binding Activity of the Mdm2 RING Domain

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Department of Biological Sciences, Columbia University, New York, NY 10027

The RING domain of Mdm2 contains a conserved Walker A or P-loop motif characteristic of nucleotide binding proteins. As has been previously shown, Mdm2 preferentially binds adenine base nucleotides and that such binding leads to a conformational change in the Mdm2 C-terminus. (Poyurovsky et al. Mol Cell. 12: 875-87, 2003). Nucleotide binding defective Mdm2 mutants are impaired in p14/ARF-independent nucleolar localization both *in vivo* and *in vitro,* and ATP-bound Mdm2 is preferentially localized to the nucleolus.

Here we present further biochemical analysis of the nucleotide-Mdm2 interaction. We confirmed the original ATP binding and specificity results using Isothermal Titration Calorimetry (ITC). Further investigation of the interaction using a series of ATP derivatives identified 2' and 3' hydroxyls of the ribose as well as the C6 amino group of the adenine base moiety as being essential for the interaction. These results further support our previous data on ATP specificity, as the C6 amino group is a unique feature of adenine. MdmX, an Mdm2 family protein with high sequence homology, similarly binds adenine nucleotides preferentially. In order to further elucidate the structural features of Mdm2 necessary for ATP interaction, we have created a series of substitution mutations in residues within the Mdm2 RING domain that were predicted to be involved in base recognition. We assayed the resulting mutant proteins for nucleotide binding, nucleotide specificity, and E3 ligase activity. Our results highlight an intriguing separability between nucleotide binding and E3 functions of the Mdm2 RING domain, indicating that this domain may be involved in several unrelated biochemical processes. (Supported by DOD propoposal #BC044468)

Conference presentations:

10th Annual Spinal Muscular Atrophy Research Group Meeting
June 10-12th, Montreal, Canada

High-Throughput Screen for Compounds that Increase SMN Protein Level

Reka Letso, Columbia University, Laboratory of Dr. Brent Stockwell

Spinal muscular atrophy (SMA) is a pediatric neurodegenerative disease in which nearly all patients have a homozygous deletion of the *survival of motor neuron 1 (smn1)* gene.

However, patients retain at least one copy of the nearly identical gene *smn2*, which cannot fully compensate for the deleted gene due to increased use of an alternative splice site. Due to the existence of this modifiable gene isoform, SMA is an exceptional candidate for small molecule intervention. Therefore, we have undertaken a high-throughput screen in patient fibroblast cells to discover small molecules which increase endogenous SMN protein levels. To date we have screened ~30,000 compounds from our unique chemical libraries which were chosen using the latest cheminformatic approaches for selection of drug-like compounds with increased likelihood of crossing the blood-brain barrier. One hit has been confirmed and further characterization of its mechanism of action may help elucidate the molecular basis of SMA.

Conclusions

In summary, we have made progress on developing an alternate high-throughput assay. In the following year we aim to test compounds identified by the *in silico* screens to find lead molecules binding to MDM2. We will then optimize the structures of these MDM2 ligands to improve potency, toxicity and selectivity. These studies may lead to the discovery of novel, selective MDM2 inhibitors that are effective treatments for breast cancers.

References

Kostic, M., Matt, T. Martinez-Yamout, M.A., Dyson, H.J. Wright, P.E., (2006) Solution structure of the Hdm2 C2H2C4 RING, a domain critical for ubiquitination of p53. J Mol Biol. *2* ,433-50.

Openeye Scientific, Santa Fe

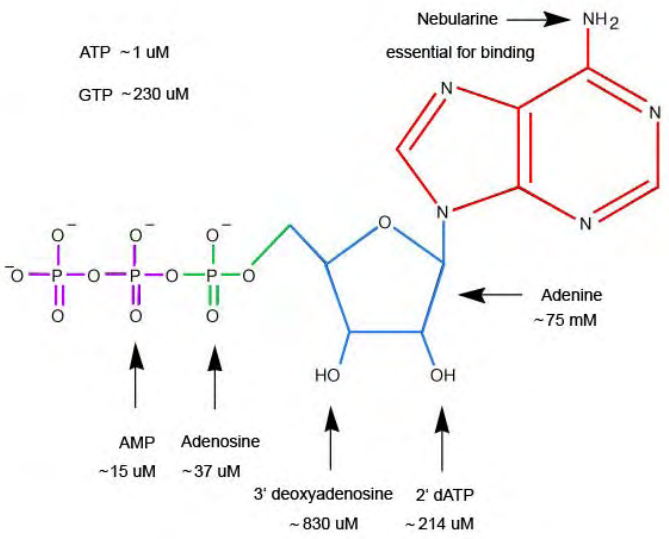
Poyurovsky, M. V., Jacq, X., Ma, C., Karni-Schmidt, O., Parker, P. J., Chalfie, M., Manley, J. L., and Prives, C. (2003). Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. Mol Cell *12*, 875-887.

Appendix/Supporting Data

(a)

Compound	K_d (M)	Std. Err.
BODIPY FL ATP- -S thioether	0.7779	0.08318
Fluorescein-12-ATP	0.142	0.03782
AMP	14.82	.06545
Adenosine	37.05	.118
Adenine	74506	42.54
3' deoxy adenosine	829.3	.0824
2' deoxyadenosine	infinite	N/A
GTP	231.1	.1841
Ribavirin	41.36	.07778
Nebularine	infinite	N/A
8-amino ATP	.3268	.08245
Arabinosyl-ATP	18.16	.2369

(b)



(c)

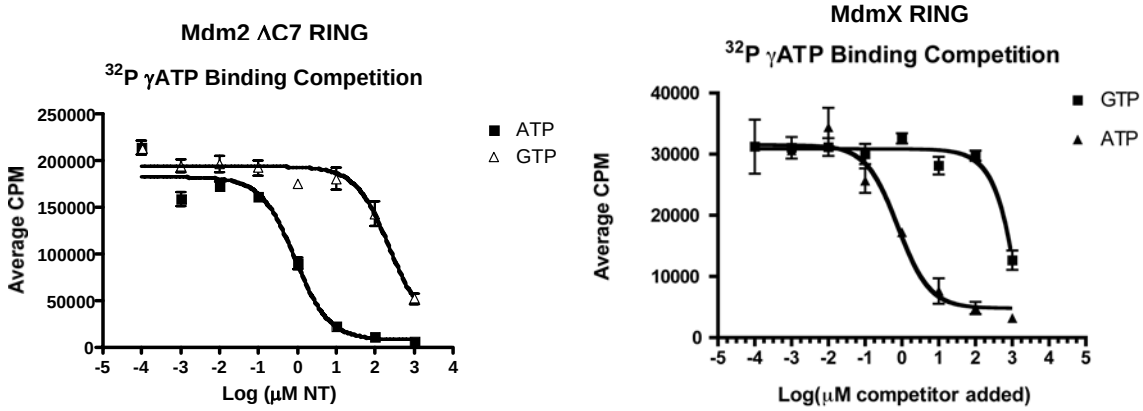


Figure 1

Mdm2 and MdmX RING domains preferentially bind adenine-based nucleotides. (a) Disassociation constants and associated standard errors determined using binding competition curves of commercially available ATP analogs (new compounds highlighted in yellow. (b) Features of ATP required for Mdm2 binding as indicated by disassociation constants (K_d) of ATP derivatives. (c) MdmX RING domain has similar affinities for ATP and GTP as Mdm2 RING domain.

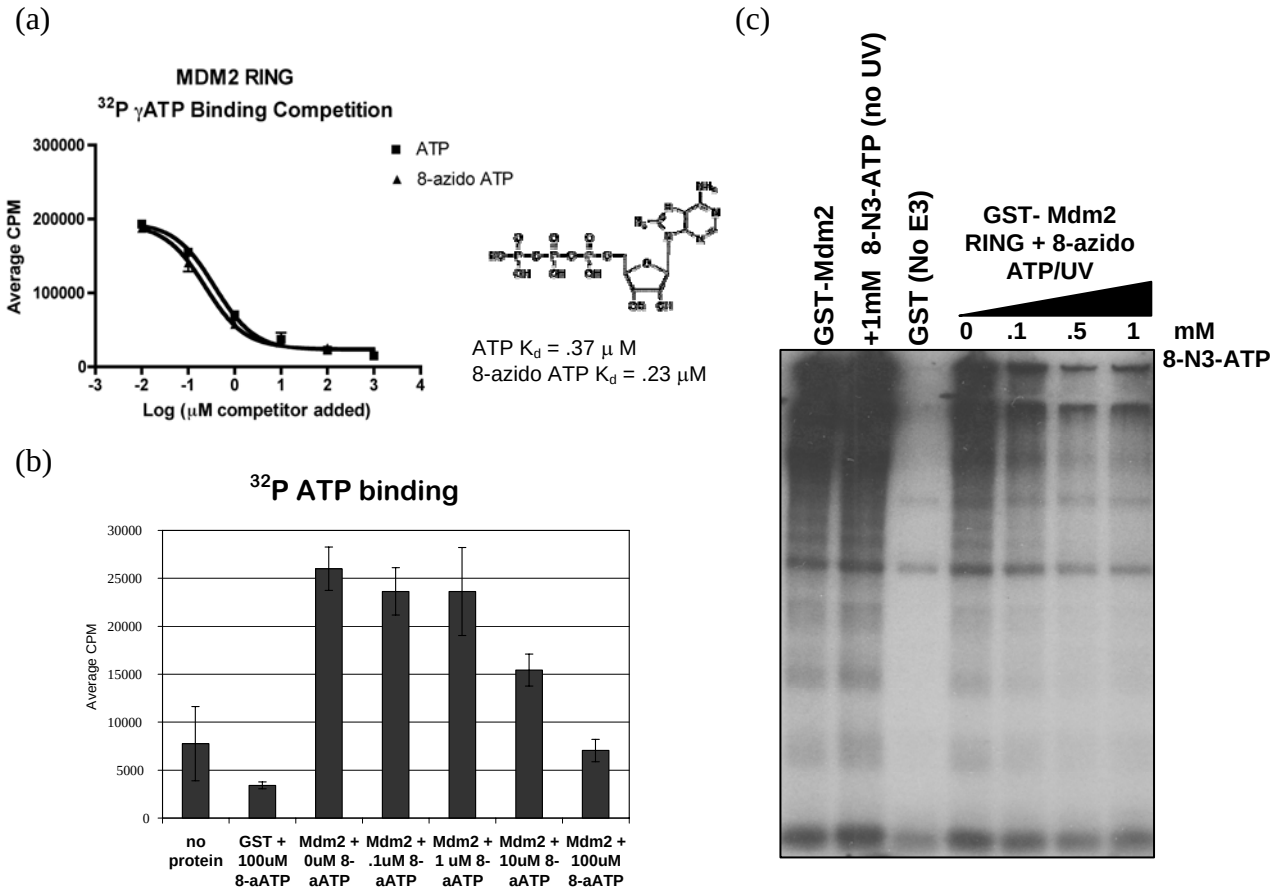


Figure 2
Crosslinking 8-azido ATP to the Mdm2 RING domain inhibits ATP binding and E3 ligase activity (a) 8-azido ATP binds Mdm2 with similar affinity as ATP. (b) Crosslinking to 8-azido ATP significantly reduces ATP binding by Mdm2. (c) Crosslinking to 8-azido ATP inhibits Mdm2 *in vitro* ubiquitin polymerization activity. All error bars represent the standard deviation of 2 replicates.

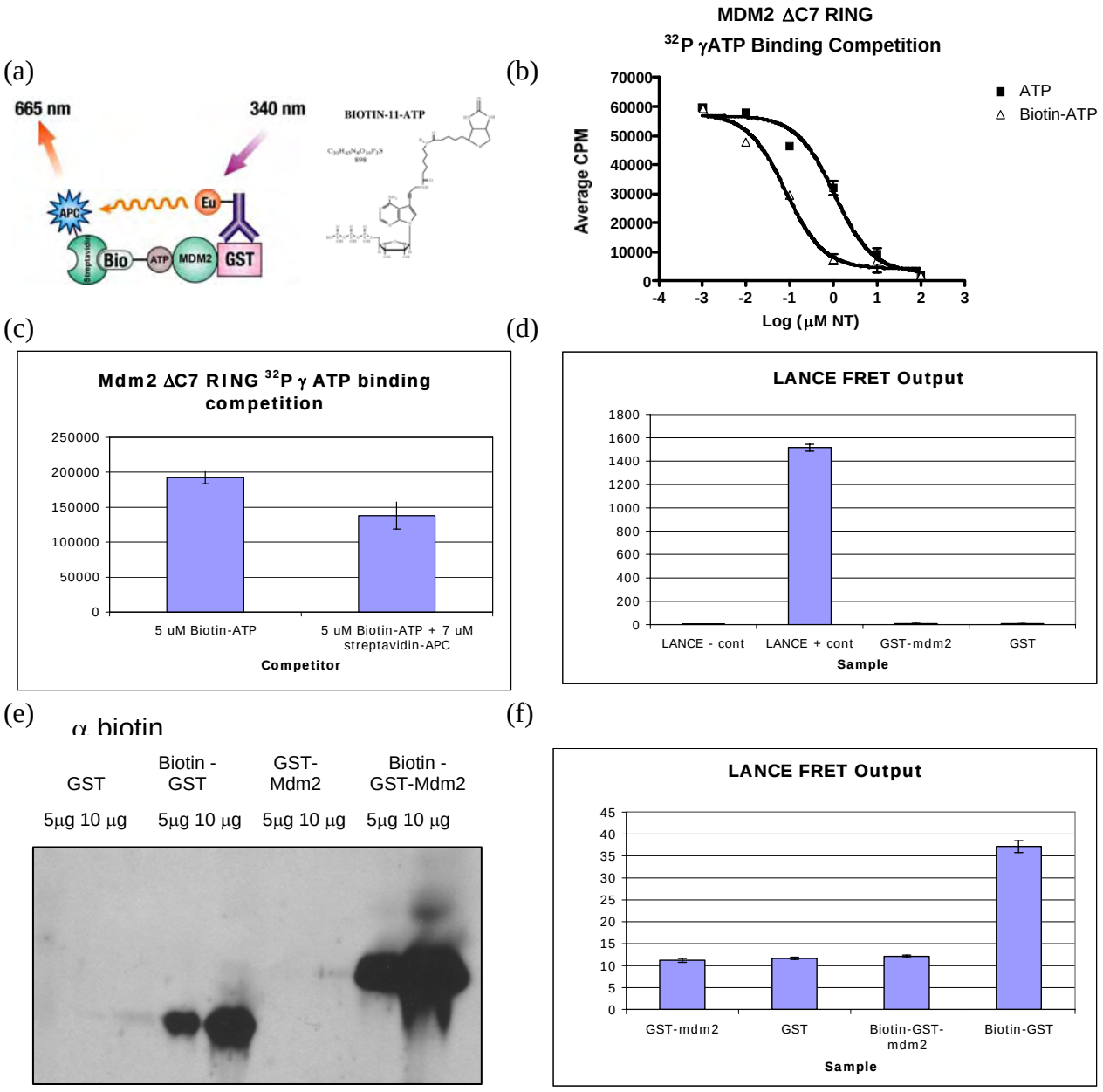


Figure 3

Design and optimization of Lance TR-FRET assay. (a) Assay design and structure of biotin-labeled ATP. (b) Competition of unlabeled ATP and Biotin-11-ATP with ^{32}P γ-ATP for binding to GST-Mdm2 ΔC7 RING. (c) Competition of Biotin-11-ATP with ^{32}P γ-ATP for binding to GST-Mdm2 ΔC7 RING is unaltered in the presence of streptavidin-APC. (d) Lance FRET assay output in the presence of GST (negative control) or GST-Mdm2 ΔC7 RING. (e) α-biotin immunoblot after biotinylation of GST and GST-Mdm2 ΔC7 RING. (f) Lance FRET output in the presence of untreated or biotinylated GST or GST-Mdm2 ΔC7 RING. All error bars represent the standard deviation of two replicates.

(a) (b)

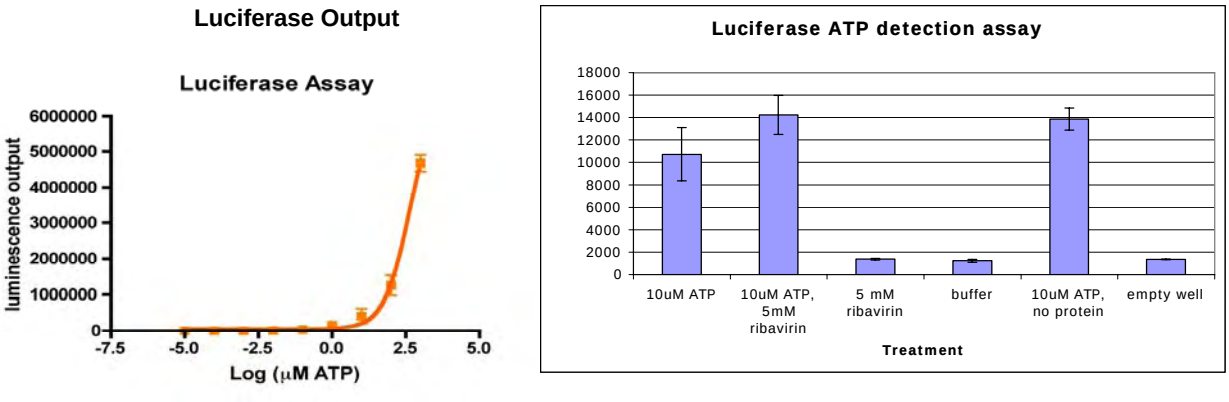


Figure 4
Luciferase ATP detection assay optimization and preliminary data (a) Luciferase assay luminscence ouput increases with ATP concentration. (b) Sample luciferase competition assay with ribavirin (Mdm2 Kd ~40μM). Error bars represent the standard deviation of three replicates.

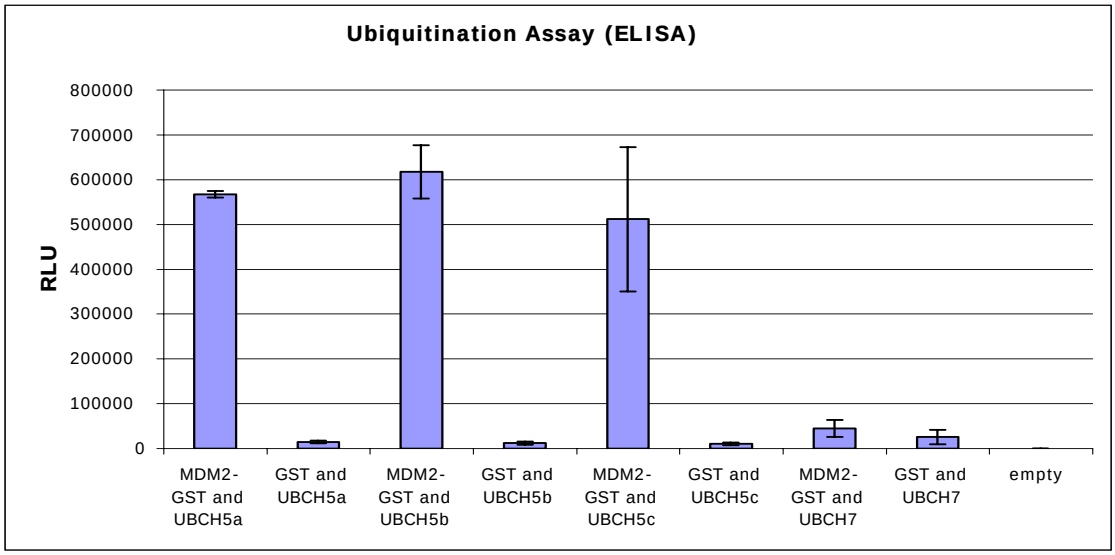


Figure 5. Mdm2 auto-ubiquitination assay. The assay works with any of the UBCH5 proteins, which are known to function with MDM2 (400-491)-GST. However, UBCH7 which has not been shown to function as an E2 with MDM2 does not work in this assay. Additionally the ubiquitination reaction was verified on a Western Blot analysis (data not shown). This assay can be used to screen large numbers of small molecules in a 384-well high-throughput format to find MDM2 E3 ligase inhibitors.

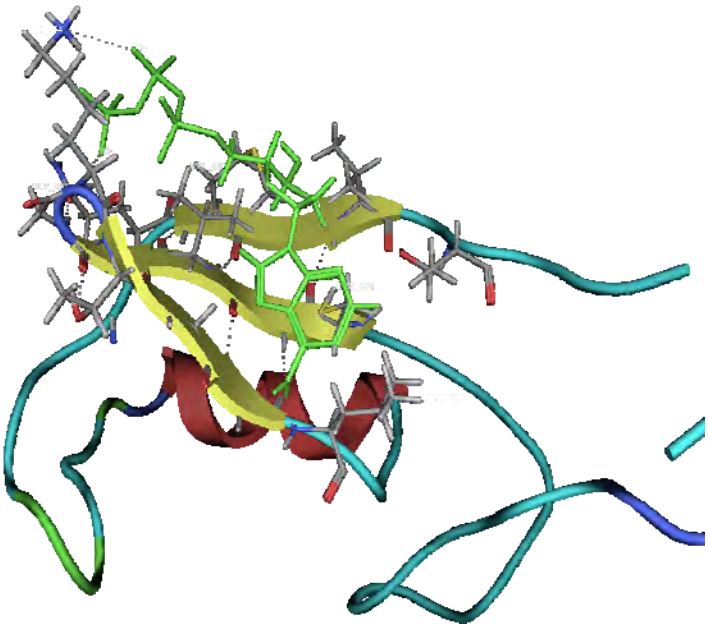


Figure 6. Proposed binding site on Mdm2 RING domain, showing K454 coordinating phosphate groups of ATP.